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Waveform Dynamics of Spermatozeugmata During the Transfer From Paternal to Maternal Individuals of *Membranipora membranacea*

M. H. TEMKIN[1,*] AND S. B. BORTOLAMI[2]

[1] *Biology Department, St. Lawrence University, Canton, New York 13617; and* [2] *Ashton Graybiel Spatial Orientation Laboratory, Brandeis University, Waltham, Massachusetts 02254*

Abstract. Analysis of standard (60 frames/s) and high-speed (200 frames/s) video records revealed that unencapsulated sperm aggregates (spermatozeugmata) of the gymnolaemate bryozoan *Membranipora membranacea* spontaneously generate at least three types of waveforms: small amplitude, large amplitude, and reverse. All three waveforms significantly differed from one another in amplitude. Additionally, small- and large-amplitude waveforms propagated from the base to the tip of axonemes, whereas the reverse waveform propagated from the tip to the base of axonemes. Small-amplitude waveforms, which were generated most frequently by spermatozeugmata in the paternal perivisceral coelom and in the water column after spawning, produced almost no curvature of the axoneme. Large-amplitude waveforms were produced by spermatozeugmata in the water column and within lophophores. Reverse waveforms were produced while spermatozeugmata moved tail-end forward through the paternal tentacles during spawning and after spermatozeugmata had contacted the intertentacular organ (ITO), a tubular structure that spermatozeugmata pass through to enter the maternal coelom and that eggs pass through to enter the seawater. The production of reverse waveforms by spermatozeugmata after reaching the ITO may be evidence for a behavioral response of bryozoan sperm to conspecific maternal individuals.

Introduction

Fertilization success for many benthic marine invertebrates is dependent on the transfer of sperm or an aggregate of sperm from males to females through the water column (see Franzén, 1956, 1998; Ryland and Bishop, 1993). Sperm aggregates may be either encapsulated (spermatophores) or unencapsulated (spermatozeugmata). The transfer of sperm, spermatophores, or spermatozeugmata from male to female conspecific benthic marine invertebrates may be influenced by numerous physical and biological factors. For example, water flow, population density, spawning synchrony, sperm chemoattractants, gamete longevity, and sperm motility are all factors that have been reported to increase or decrease fertilization success by altering the probability that sperm will find maternal individuals (see Ryland and Bishop, 1993; Levitan, 1995).

In species that transfer sperm from males to females through the water column, fertilization success may ultimately depend on sperm motility and behavior once a conspecific female has been approached or contacted. For example, sperm become attached to external maternal structures where they wait for eggs to be spawned, as in some sabellid polychaetes (*e.g.,* Daly and Golding, 1977; Rouse, 1996) and some bivalves (*e.g.,* Ó Foighil, 1985, 1989). In other species, sperm enter maternal individuals to fuse with eggs internally, as in some hydroids (see Miller, 1983), the sea cucumber *Leptosynapta clarki* (Sewell and Chia, 1994), the colonial ascidian *Diplosoma listerianum* (Bishop and Ryland, 1991; Burighel and Martinucci, 1994a, b), phoronids (see Zimmer, 1991), and the gymnolaemate bryozoan *Membranipora membranacea* (Temkin, 1994). Yet few observations have been made on how sperm attach to or enter conspecific females, or on how sperm locate eggs prior to fertilization.

Among gymnolaemate bryozoans, one of the most detailed descriptions of sperm transfer has been reported for *Membranipora membranacea* (Temkin, 1994). Zooids of

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* To whom correspondence should be addressed. E-mail: mtemkin@stlawu.edu

Abbreviation: ITO, intertentacular organ.

M. membranacea colonies typically are functionally simultaneous hermaphrodites, and sperm in spermatozeugmata are transferred from paternal to maternal zooids through the water column (Temkin, 1994). Like those of other gymnolaemate bryozoans, the spermatogonia of *M. membranacea* migrate into the perivisceral coelom from the peritoneum of the body wall, or funiculus (a network of strand-like elements of the circulatory system), and form syncytial masses of spermatocytes around cytoplasmic masses or cytophores (see Reed, 1991; Franzén, 1998). Cells of a cytophore disassociate at the end of spermiogenesis in most gymnolaemates, but in species such as *M. membranacea*, sperm remain together, adhering to one another to form a spermatozeugma.

Gymnolaemate spermatozeugmata are aggregates of 32 or 64 euspermatozoa in which the cells are arranged parallel to one another in a hexagonal array, with all the heads at one end of the aggregate and all of the tails at the other (Bonnevie, 1907; Franzén, 1956, 1998; Zimmer and Woollacott, 1974) (Fig. 1). Spermatozeugmata of gymnolaemate bryozoans are held together by viscomechanical forces that tightly adhere sperm to one another along the head region, the tail-end half of the midpiece region, and almost all of the tail region (see Franzén, 1956, 1998; Temkin, 1994). The two regions of a spermatozeugma where sperm do not adhere to each other are the head-end half of the midpiece and the tip of the tail (Fig. 1).

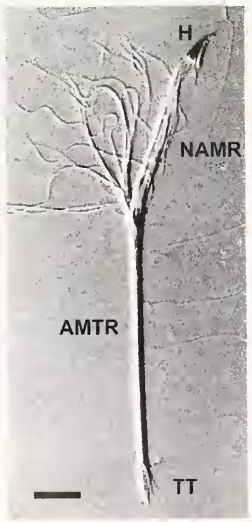

Figure 1. Differential interference contrast image of a partially disassociated spermatozeugma adhering to a glass slide. The head-end half of the spermatozeugma consists of the elongate head regions (H) that are adhering to one another and the nonadhering portions of the midpiece regions (NAMR). The tail-end half consists of the adhering portions of the midpiece and tail regions (AMTR) and the tips of the tails (TT). Sperm that have become partially disassociated from the aggregate generate a variety of waveforms. Scale bar = 12 μm.

Sperm of *M. membranacea* within spermatozeugmata generate waveforms, and movements of the midpiece regions may produce some of the motive forces required for both spawning and entry into maternal individuals (Temkin, 1994). Spermatozeugmata of *M. membranacea* are motile in the paternal coelom, like sperm of other gymnolaemate bryozoans (Marcus, 1938; Silén, 1966; Zimmer and Woollacott, 1974; Temkin, 1994). Prior to spawning, spermatozeugmata of *M. membranacea* become positioned in the perivisceral coelom between the body wall and the distomedial side of the pharynx, so that their tail ends are oriented toward the pore leading into the lophophoral coelom of the two distomedial tentacles (Temkin, 1994). Cilia located near the pore may help spermatozeugmata enter the lophophoral coelom of the distomedial tentacles (R. Zimmer, University of Southern California, pers. comm.). Spermatozeugmata move through the two distomedial tentacles, emerging tail-end first into the exhalant feeding currents of colonies. During their passage through the tentacle lumen, spermatozeugmata appeared to be pushed by waveforms produced in the midpiece region (Temkin, 1994).

In the laboratory, spermatozeugmata become quiescent after they are spawned, until they are drawn into the lophophores of conspecifics by colony feeding currents (Temkin, 1994). Spermatozeugmata retained within the lophophores of conspecifics often produce strong undulatory movements in the midpiece region (Temkin, 1994). While in the lophophore, spermatozeugmata may move headfirst into maternal individuals through the intertentacular organ (ITO). The ITO is a tubular secondary sex structure formed by the basal fusion of the two distomedial tentacles (see Silén, 1966; Reed, 1991). The ITO serves not only as the entry organ for spermatozeugmata, but also as the spawning organ for eggs (*e.g.*, Silén, 1966; Temkin, 1994). Temkin (1994) reported that spermatozeugmata that entered the distal pore of the ITO appeared to stop their undulatory movements and were drawn into the ITO. After entering maternal individuals, sperm of a spermatozeugma disassociate and migrate to the surface of the ovary, although the exact sequence of these two events is uncertain. In *M. membranacea*, sperm-egg fusion occurs during or shortly after ovulation and is monospermic (Temkin, 1994).

In this paper, we compare the waveforms generated by spermatozeugmata of *M. membranacea* within the paternal coeloms (perivisceral and lophophoral), in seawater after spawning, and within the lophophores of conspecifics. We describe three types of waveforms (small amplitude, large amplitude, and reverse) that are spontaneously generated by spermatozeugmata of *M. membranacea* during the transfer of aggregates from paternal to maternal zooids. In addition,

we relate the functional significance of the waveforms to the structure of spermatozeugmata and to sperm transfer.

Materials and Methods

Colonies of *Membranipora membranacea* Linnaeus, 1767, were collected from waters near the Friday Harbor Laboratories (FHL), San Juan Island, Washington, and the Darling Marine Center (DMC), Walpole, Maine (for a phylogeographic analysis of these populations, see Schwaninger, 1999). The movements of spermatozeugmata within paternal coeloms, in seawater after spawning, and within the tentacle crown of maternal lophophores were videorecorded. Videorecordings were made using Panasonic cameras (60 frame/s) mounted on either Zeiss (FHL) or Olympus (DMC) research compound microscopes. Some *M. membranacea* colonies collected at the DMC were transported to Harvard University, where videorecordings were made using an NAC HSV-200 video camera (200 frames/s) mounted on a Zeiss Photo III compound microscope. To view spermatozeugmata within paternal coeloms and maternal lophophores, we examined "one-zooid-row" preparations that were placed on their sides in small petri dishes (Temkin, 1994). To make recordings of spermatozeugmata outside of the paternal coelom (seawater), individual spermatozeugma that had been recently spawned were removed from dishes containing one-zooid-row preparations and placed in depression slides containing 50 to 100 μl of 0.2-μm-filtered seawater (FSW) at room temperature.

Recorded sequences were viewed frame by frame to analyze the waveforms produced by spermatozeugmata during each trial. Waveforms produced by spermatozeugmata were distinguished based on waveform amplitude and direction of waveform propagation. The amplitudes of 10 waves for each of the three recognized waveforms were determined using the computer program Image Tool ver. 3.00 (University of Texas, http://ddsdx.uthscsa.edu/dig/itdesc.html), calibrated with an image of a stage micrometer, to measure digitized images of high-speed video frames. Systat 6 was used to calculate an analysis of variance (ANOVA), and Bonferroni adjusted pairwise comparisons were made to determine statistical differences among waveform amplitudes. The frequency of wave generation was measured in 25 reverse-waveform events for spermatozeugmata within the paternal coelom and calculated as the number of waves per second. In addition, waveform patterns generated by DMC spermatozeugmata in the paternal visceral coelom ($n = 10$) and in seawater after spawning ($n = 10$) were compared by determining the frequency and duration of reverse-waveform events. To be included in the comparison, spermatozeugmata had to have a video record of at least 20 s. Event frequencies were calculated as the number of waveform events per minute. Durations of reverse-waveform events during an individual trial were averaged. Two-sample t tests were calculated using Systat 6 to compare the frequencies and durations of reverse-waveform events between spermatozeugmata in the paternal coelom and water column.

Results

Waveform types

Spermatozeugmata of *Membranipora membranacea* from both the DMC and FHL generated three types of waveforms: small amplitude, large amplitude, and reverse. The amplitudes of these waveforms were greatest in the nonadhering portions of the midpiece regions of spermatozeugmata. The adhering portions of the midpiece and tail regions showed no apparent curvature during the generation of any of the three waveforms (Figs. 2, 3, and 4). An ANOVA and pairwise comparisons of means revealed that the amplitudes of the three waveforms significantly differed from one another (Table 1). Small-amplitude waveforms consisted of waves with amplitudes of 1.9 $\pm$ 1.6 μm (mean $\pm$ standard deviation, $n = 10$) that were generated from the base to the tip of axonemes (*i.e.*, head to tail). Sperm within a spermatozeugma typically produced small-amplitude waveforms asynchronously. Consequently, small-amplitude waveforms were difficult to observe unless sperm in an aggregate produced this type of waveform synchronously (Fig. 2). Small-amplitude waveforms produced almost no curvature in spermatozeugmata as waves moved along axonemes. Large-amplitude waveforms also propagated from the base to the tip of axonemes (Fig. 3), with amplitudes of 11.1 $\pm$ 3.0 μm ($n = 10$). During the generation of large-amplitude waveforms, spermatozeugmata undulated and rotated around their long axis. Reverse waveforms had amplitudes of 7.0 $\pm$ 2.4 μm ($n = 10$) and propagated from the tip to the base of axonemes (*i.e.*, tail to head) (Figs. 4 and 5); that is, reverse waveforms were propagated along the axoneme in a direction opposite to that of small- and large-amplitude waveforms. During reverse-waveform events in the paternal visceral coelom, waves were generated with a frequency of 11.0 $\pm$ 0.5 waves/s ($n = 25$). The generation of reverse waveforms was distinguished by the development of prominent bends near the junctions of (1) the heads and midpieces and (2) the nonadhering and adhering portions of midpieces (Figs. 4 and 5). The curvature in the anterior portions of a spermatozeugma during a reverse-waveform event causes the aggregate to bend over, giving a spermatozeugma the distinctive appearance of a question mark.

Location-specific waveform generation

Spermatozeugmata generated different patterns of waves depending on whether they were located in the paternal coeloms (visceral and lophophoral), water column, or ma-

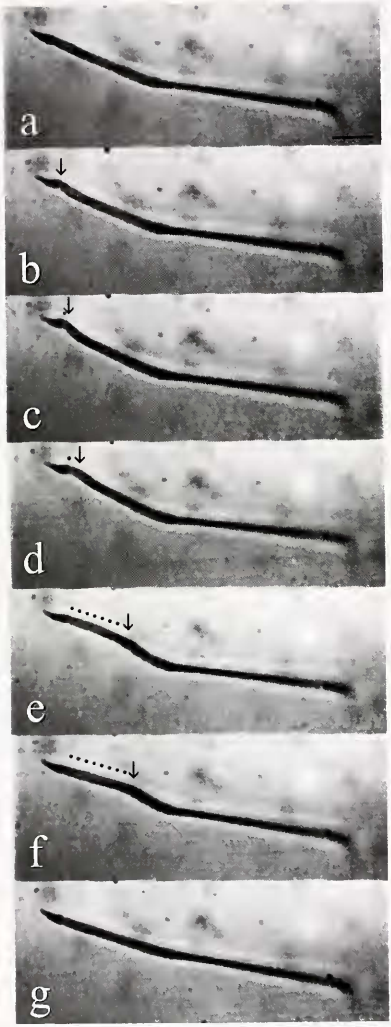

Figure 2. Synchronous production of a type I waveform by *Membranipora membranacea* spermatozeugma in seawater after spawning. (a) Asynchronous waveform production. (b–f) Propagation of wave (arrow) from the base to tip of axonemes. (g) Wave enters region of midpiece in which sperm are tightly adherent to each other and is no longer visible. Images are 10 ms apart. Dots indicate the propagation of the wave along the aggregate. The first dot in each series marks the position of the wave in (b). Scale bar = 30 μm.

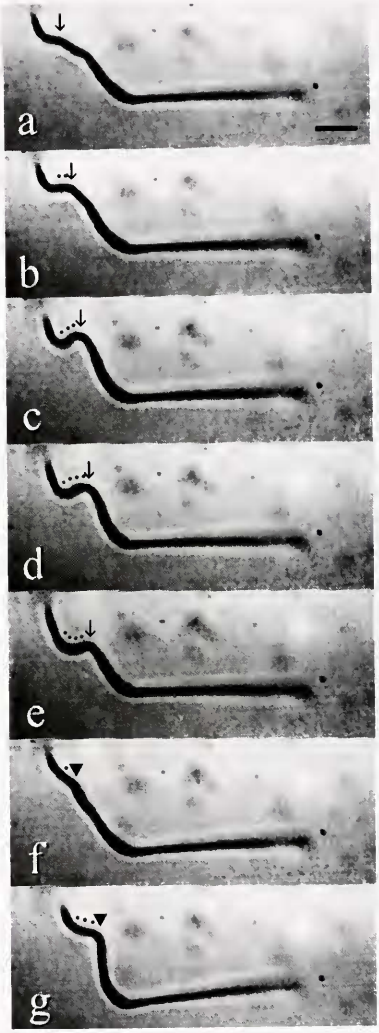

Figure 3. Generation of a type II waveform by *Membranipora membranacea* spermatozeugma in seawater after spawning. (a) A wave (arrow) forming just posterior to the heads. (b–e) The wave (arrow) propagating toward the tail end of the aggregate. (f, g) Generation of a second wave (arrowhead) after the first wave is no longer visible. Dots indicate the propagation of first wave along the aggregate. Images are 10 ms apart. Scale bar = 30 μm.

ternal lophophore. In the paternal visceral coelom and water column, spermatozeugmata generated predominantly the small-amplitude waveform, with periodic reverse-wave-form events. Spermatozeugmata in the water column also sporadically generated a short series of large-amplitude waveforms. Two-sample *t* tests revealed significant differ-

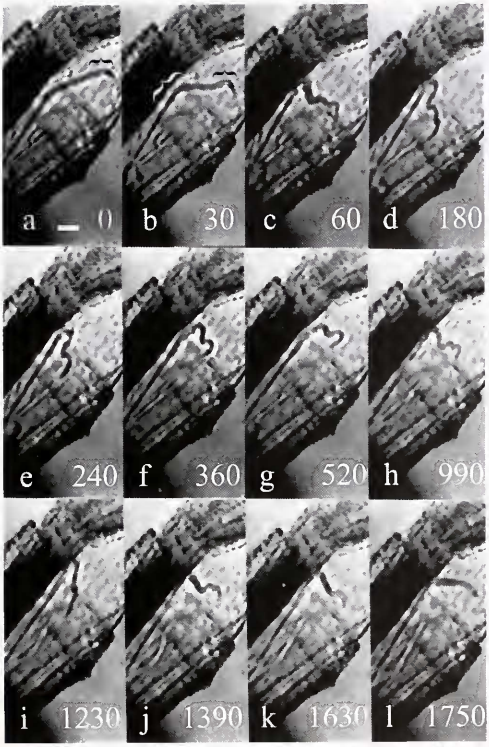

Figure 4. Variation in the conformation of a *Membranipora membranacea* spermatozeugma within paternal coelom during a 1750-ms reverse-waveform event. Values in lower right-hand corner of each image show elapsed time, from 0 to 1750 ms. (a, b) During the initiation of a reverse-waveform event, the spermatozeugma develops strong curvature near the junction of the heads and midpieces (black bracket) and near the junction of the nonadhering and adhering portions of the midpieces (white bracket). (c–j) Production of reverse waveforms bends the spermatozeugma into the shape of a cane or question mark. (k, l) Spermatozeugma returns to generating type I waveforms and a nearly linear conformation. Scale bar = 25 μm.

ences in the event frequencies (df = 18, $t = -3.04$, $P < 0.001$) and durations (df = 18, $t = -2.11$, $P < 0.05$) of reverse-waveform events between spermatozeugmata in the paternal visceral coelom and ones in the water column (Fig. 6). The reverse waveform was produced almost twice as often by spermatozeugmata in the paternal visceral coelom ($n = 10$, 9.4 ± 0.7 events/m) as by spermatozeugmata in the water column ($n = 10$, 5.6 ± 1.0 events/min). In addition, reverse-waveform events lasted about 1.5 times longer for spermatozeugmata in the paternal visceral coelom ($n = 10$, 0.94 ± 0.10 s) than for those in the water column ($n = 10$, 0.64 ± 0.11 s). During the production of reverse waveforms in the paternal perivisceral coelom and in seawater after spawning, spermatozeugmata were not observed to move in either a head-forward or a tail-forward direction.

Spermatozeugmata spent about 2.0 s in the lophophoral coelom during spawning. While traveling through the two distomedial tentacles into the external seawater, they generated reverse waveforms (Fig. 7). One sperm aggregate stopped producing the reverse waveform as it emerged from the tentacle and remained with its head end within the lophophoral coelom of the tentacle for about 10 s until reverse waveforms were generated again. With resumption of reverse-waveform production, the spermatozeugma pushed itself out of the tentacle.

Spawned spermatozeugmata were commonly swept into the lophophores of conspecifics by feeding currents. Most spermatozeugmata passed quickly through the lophophores without altering their waveform dynamics. However, when spermatozeugmata remained within the lophophores, they typically produced large-amplitude or reverse waveforms either continuously or periodically. Many of the spermatozeugmata generating large-amplitude waveforms escaped from the lophophores into the exhalant current of colonies. Spermatozeugmata generating large-amplitude waveforms were able either to swim directly out of the lophophores or to enter into the exhalant current stream of the lophophores. In one case, a spermatozeugma generating large-amplitude waveforms moved from the pharynx of a zooid, out through the mouth, and back into the lophophore. In other cases, spermatozeugmata within lophophores became positioned with their head ends at the distal pore of the ITO. Once their head ends contacted the distal surface of the ITOs, the

Table 1

Summary of statistical tests to determine differences among waveform amplitudes

Analysis of variance

Source	Sum-of-squares	df	Mean-square	*F*-ratio	*P*
Waveform type	399.29	2	199.64	34.60	0.000
Error	155.82	27	5.77		

Matrix of pairwise mean differences (below diagonal) and probabilities (above the diagonal)*

Waveform type	Small	Large	Reverse
Small		0.000	0.000
Large	8.9		0.003
Reverse	4.9	−4.0	

* Mean differences calculated using a mean squared error (MSE) model of 5.77 with 27 degrees of freedom; probabilities calculated using a Bonferroni adjustment.

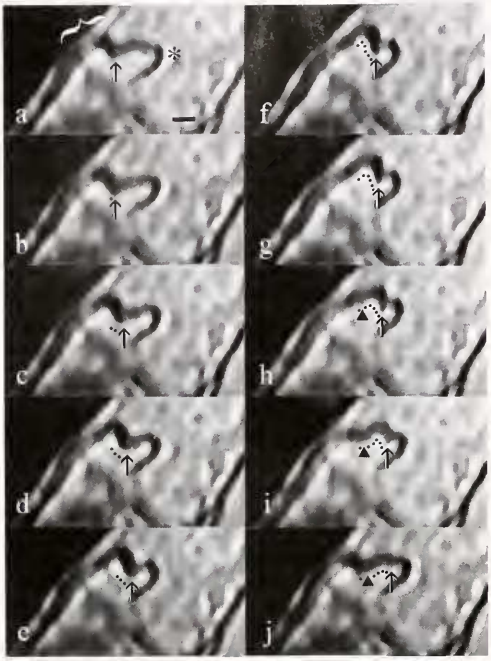

Figure 5. Propagation of reverse waveforms along the axonemes from the tips to the bases in a *Membranipora membranacea* spermatozeugma within the paternal coelom. Images are 10 ms apart. (a–j) The movement of a reverse waveform (arrow) from the midpoint of the nonadhering midpiece region toward the head end of the spermatozeugma. The white bracket and * in (a) indicate the curvature of the spermatozeugma occurring near the junction of the nonadhering and adhering portions of the midpieces and posterior to the heads, respectively. (h–j) A second wave (black arrowhead) becomes apparent and propagates toward the head end of the aggregate. The first dot of each tracking line indicates the original position of the first wave in (a). Scale bar = 20 μm.

spermatozeugmata altered their waveform dynamics to generate reverse waveforms within about 100 ms and attempted to enter the ITOs. The production of reverse waveforms bent the head ends of the spermatozeugmata toward the distal pore of the ITO (Fig. 8). Spermatozeugmata could not be observed after they entered ITOs because of cilia that line the lumen of the ITO. Consequently, we could not determine the waveforms produced by spermatozeugmata inside of ITOs.

To determine if the change from small-amplitude or large-amplitude waveform to reverse waveform after a spermatozeugma contacted an ITO was simply a touch response, the waveform dynamics of 25 spermatozeugmata were observed before and after they contacted a glass surface. In 18 cases, spermatozeugmata were producing a small-amplitude waveform when they contacted the substrate. Seventy-eight percent (14 of 18) of these spermatozeugmata continued to generate a small-amplitude waveform or a small-amplitude waveform with periodic reverse-waveform events. The remaining 22% (4 of 18) initially continued to produce a small-amplitude waveform, but later changed to reverse waveforms. Seven spermatozeugmata contacted the substrate during a reverse-waveform event, and all of them continued to generate reverse waveforms. Consequently, the change in waveform does not seem to be simply a touch response.

Discussion

Spermatozeugmata develop in a group of animals that is diverse in phylogeny and reproductive biology. For example, in addition to occurring in bryozoans, spermatozeugmata have been reported in marine and freshwater oligochaetes (see Ferraguti, 1983), gastropods (see Buckland-Nicks *et al.*, 1999, 2000), marine and freshwater bivalves (*e.g.*, Ó Foighil, 1985, 1989; Lynn, 1994; Jespersen *et al.*, 2001, 2002), insects (*e.g.*, Sahara and Kawamura, 2002), and fish (see Jamieson, 1991; Hayakawa *et al.*, 2002a). Marine and freshwater bivalves and some fish are similar to gymnolaemate bryozoans in that they spawn their spermatozeugmata into the water column. Gastropods, insects, and most fish transfer their spermatozeugmata directly from the male into the female reproductive tract using a form of copulation. Oligochaetes use a mechanism of pseudocopu-

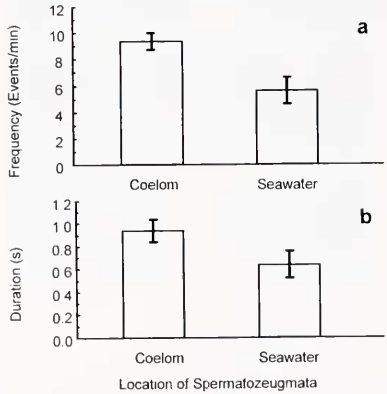

Figure 6. Event frequency (a) and duration (b) of reverse waveforms produced by *Membranipora membranacea* spermatozeugma within the paternal coelom ($n = 10$) and seawater ($n = 10$). Two sample t tests demonstrate that spermatozeugmata within the paternal coelom produce significantly more (df = 18, $t = -3.04$, $P < 0.001$) and longer (df = 18, $t = -2.41$, $P < 0.05$) reverse waveform events than spermatozeugmata in seawater.

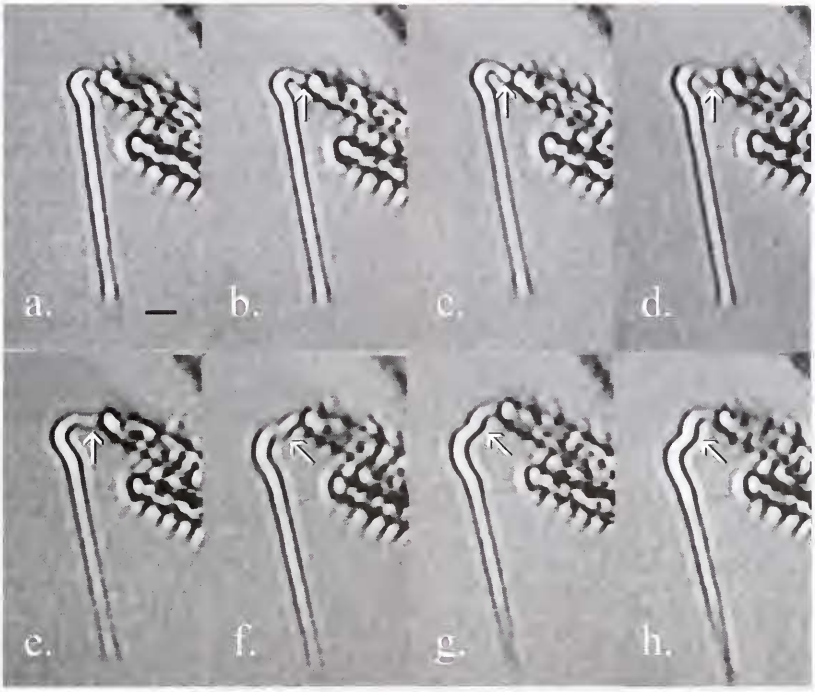

Figure 7. Spermatozeugma of *Membranipora membranacea* crawling out of a paternal tentacle tail-end forward by producing a reverse waveform. Images are 20 ms apart. Arrows indicate the position of the wave along the aggregate. Scale bar = 20 μm.

lation to deliver spermatozeugmata to the spermatheca of mating partners. The spermatozeugmata produced by many species of marine and freshwater oligochaetes, marine bivalves, gastropods, insects, and fish differ from the sperm aggregates of *Membranipora membranacea* in that they consist of dimorphic sperm (*e.g.,* Ferraguti *et al.,* 1989; Healy and Jamieson, 1993; Buckland-Nicks *et al.,* 2000; Jespersen *et al.,* 2001, 2002; Hayakawa *et al.,* 2002a; Sahara and Kawamura, 2002). One type of sperm, euspermatozoa, fertilizes eggs; the other type, paraspermatozoa, does not fuse with eggs, but instead is thought to enhance the fertilization success of euspermatozoa through a variety of mechanisms, including preventing sperm from other conspecific males from fertilizing eggs (see Buckland-Nicks *et al.,* 1999; Hayakawa *et al.,* 2002b; Sahara and Kawamura, 2002).

The production of spermatozeugmata by *M. membranacea* may increase fertilization success in three ways. First, "packaging" sperm together may reduce the loss of sperm during the transfer from paternal to maternal individuals (*e.g.,* Braidotti and Ferraguti, 1982; Ó Foighil, 1989; Lynn, 1994; Jespersen and Lutzen, 2001; Hayakawa *et al.,* 2002b). Among gymnolaemate bryozoans, *M. membranacea* has an uncommon reproductive biology (see Reed, 1991; Temkin and Zimmer, 2002). In most gymnolaemate bryozoans, the maternal zooids produce only one or a few eggs during each reproductive period; these are spawned to an external brood site, where they develop into lecithotrophic larvae, either coronate or pseudocyphonautes. In contrast, maternal zooids in species of *Membranipora* and *Electra,* as well as some species of *Alcyonidium, Farella,* and *Hypophorella,* produce many small, yolk-poor oocytes that are spawned into the water column, where they develop into planktotrophic cyphonautes. In *M. membranacea,* the synchronous development of groups of oocytes may result in the presence of as many as 25 ovulated primary oocytes in the perivisceral coelom at one time (Hageman, 1983). Consequently, the maternal zooids of *M. membranacea* likely need to acquire more sperm than most gymnolaemate bryozoans to fertilize their eggs. By transferring aggregates of sperm, the entry of one spermatozeugma into a maternal zooid delivers 64 sperm cells. In other gymnolaemate spe-

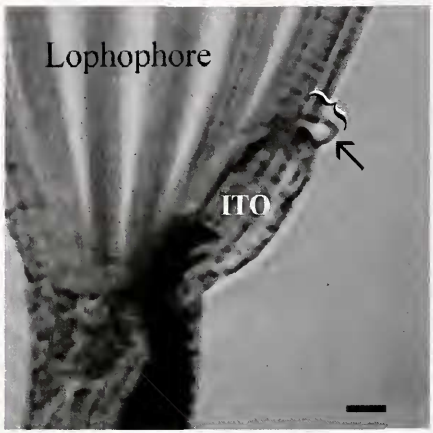

Figure 8. Spermatozeugma of *Membranipora membranacea* generating reverse waveforms after contacting the distal surface of an intertentacular organ (ITO) of a maternal individual. The anterior portion of the spermatozeugma (arrow) is projecting out of the lophophore between the two distomedial tentacles. The head end of the aggregate is in contact with the distal surface of the ITO. The anterior end of the spermatozeugma is strongly curved (bracket) due to the generation of reverse waveforms. As a result of the bend in the anterior portion of the aggregate, the spermatozeugma is able to enter the distal pore of the ITO. Scale bar = 50 μm.

cies that may face similar pressures to fertilize their eggs, spermatozeugmata occur in species of *Electra,* but have not yet been reported for species of *Alcyonidium, Farella,* and *Hypophorella.*

Second, the formation of spermatozeugmata has been suggested to increase fertilization success by increasing sperm longevity (Lynn, 1994). Currently, there are few data with which to assess the importance of spermatozeugmata on the longevity of bryozoan sperm after spawning. Manríquez *et al.* (2001) reported that the fertile half life of spawned *Celleporella hyalina* sperm at a concentration of 10 to 10^2 cells ml^{-1} was about 1.2 h. In another study, spermatozeugmata of *M. membranacea* were observed to remain motile for 36 h, although the ability of these sperm to fertilize eggs was not determined (Temkin, 1991).

Third, in some species, the formation of spermatozeugmata facilitates fertilization success by enhancing sperm motility. In organisms with dimorphic sperm, motile paraspermatozoa may transport euspermatozoa to sites of fertilization in oligochaetes and some gastropods (Ferraguti *et al.,* 1988; see Buckland-Nicks *et al.,* 2000). In contrast, the flagellar beat of euspermatozoa generates the motility associated with the spherical spermatozeugmata of the bivalve *Anodonta grandis* (Lynn, 1994). The movements of euspermatozoa contained within spermatozeugmata of *M. membranacea* are restricted because cells are bound together along most of their lengths by viscomechanical forces. The adherence of sperm to one another within a spermatozeugma of *M. membranacea* establishes a structural and functional division of the aggregate into head and tail halves. The head-end half consists of the head regions that are tightly adherent to each other and the nonadhering portions of the midpiece region. The head-end half is the region of a spermatozeugma in which waveforms achieve their greatest amplitude and where the aggregate undergoes conformational changes that create a strong curvature during the production of waveforms generated from the tip to the base of axonemes (*e.g.,* during reverse-waveform events). The tail-end half of a spermatozeugma forms a stiffened, rodlike region consisting of the adhering portions of the midpiece and tail regions. In fact, no discernible curvature was observed during the generation of any waveform in the tail-end half of spermatozeugmata during this study. Consequently, the forces that move spermatozeugmata of *M. membranacea* appear to be generated in the head-end half of the aggregates rather than along the entire length of the midpiece and tail regions.

Sperm in spermatozeugmata of *M. membranipora* may generate at least four types of waveforms. Here, we observed the spontaneous generation of three waveforms: small amplitude, large amplitude, and reverse. A fourth waveform type, in which the head-end halves of spermatozeugmata produce an effective stroke-recovery stroke movement similar to that of a cilium, can be induced by placing spermatozeugmata in seawater containing elevated levels of either Ca^{2+} or K^+ (Temkin, 2002). During small- and large-amplitude waveform events, waveforms are generated from the base to the tip of axonemes. In contrast, during reverse and cilium-like movements, waveforms are generated from the tip to the base of axonemes.

Modulating the direction of waveform propagation along the axoneme is a rare phenomenon among animal sperm (Afzelius, 1982; Baccetti *et al.,* 1989). The ability to naturally reverse the direction of waveform propagation has been reported for the sperm of the polyclad turbellarians *Notoplana atomata, Polyposthia similis,* and *Leptoplana tremellaris* (Hendelberg, 1965, 1983), the parasitic polychaete *Myzostomum cirriferum* (Afzelius, 1982, 1983), and the tephritid flies *Ceretitis capitata, Dacus oleae,* and *D. dorsalis* (Baccetti *et al.,* 1989). Among these organisms, modulating the direction of waveform propagation is not specific to any one organization of the axoneme or mode of sperm transfer. The sperm of *M. membranacea* have a 9 + 2 axoneme (Zimmer and Woollacott, 1974) and are transferred to maternal individuals through the water column aggregated into spermatozeugmata. The threadlike sperm of *N. atomata, P. similis,* and *L. tremellaris* contain two 9 + 1 axonemes and are copulated directly into the reproductive tract of females (Hendelberg, 1965, 1983). In *M. cirriferum,* sperm have a 9 + 0 axoneme and are packaged into spermatophores that are reciprocally transferred to the epidermis

of hermaphroditic partners (Afzelius, 1983). The sperm of tephritid flies have a 9 + 9 + 2 axoneme and are directly copulated into the female reproductive tract as spermatodesmata, aggregates partially encapsulated around the head ends of the sperm (Baccetti *et al.*, 1989).

In *M. membranacea*, the motility of a spermatozeugma is dependent on the synchronous generation of waveforms by most sperm of the aggregate. At spawning, spermatozeugmata move through the paternal tentacles tail-end first, generating reverse waveforms that are translated along axonemes from tip to base. To explain how spermatozeugmata move through tentacles during spawning, we propose a crawling model. While in the tentacle lumen, spermatozeugmata generate reverse waveforms that push against the internal walls of the tentacles (Fig. 9). As reverse waveforms are generated from the tip to the base of the axoneme, spermatozeugmata move in a tail-forward direction. Since the generation of reverse waveforms did not seem to move spermatozeugmata in the paternal perivisceral coelom or in seawater, contact with a surface may be important for the effectiveness of reverse waveforms in moving spermatozeugmata. In contrast, generation of large-amplitude waveforms and cilium-like movements propels spermatozeugmata with the head-end forward without needing to push against a surface (Temkin, 2002). It seems likely that the generation of either large-amplitude waveforms or cilium-like movements pulls spermatozeugmata into ITOs. During this process, the adhering portions of the midpiece and tail appear rigid, which may have caused Temkin (1994) to report that the undulatory movements of spermatozeugmata stop after the head ends enter the ITO. The actual waveforms used by *M. membranacea* spermatozeugmata after entering ITOs remain to be determined, because the view of spermatozeugmata inside an ITO is obscured by the cilia that line the lumen of the organ.

The waveform patterns generated by sperm of *M. membranacea* in spermatozeugmata change after spawning into seawater. Temkin (1994) reported that spermatozeugmata seemed to become quiescent shortly after spawning. Unlike the quiescence of sea urchin (Gibbons, 1980), tunicate (Brokaw, 1984), and polychaete (Pacey *et al.*, 1994) sperm, in which the beat of the flagellum ceases, the apparent quiescence in *M. membranacea* spermatozeugmata is caused by the decrease in the frequency and duration of reverse waveforms after spermatozeugmata enter the water column. Evidence for a similar change in waveform dynamics for spermatozeugmata of *Electra pilosa* may be contained in a paper by Marcus (1926). Marcus (1926) reported that sperm of *E. pilosa* became immobile and died shortly after being transferred into seawater. Decreasing the frequency and duration of reverse-waveform events after spawning may allow spermatozeugmata to conserve energy and may increase the longevity of sperm in the water column. Ho and Suarez (2003) have shown that increases in wave amplitude in bull sperm require increases in ATP consumption. Since small-amplitude waveforms produce almost no curvature of the axoneme compared to large-amplitude and reverse waveforms, small-amplitude waveforms may require less energy to produce than the other waveforms. In *M. membranacea*, the actual consumption of ATP by spermatozeugmata inside and outside of the paternal visceral coelom remains to be determined.

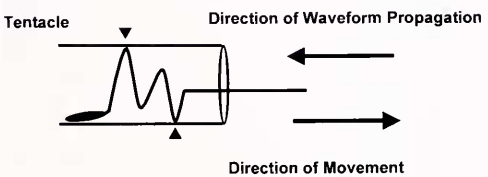

Figure 9. Model of spermatozeugma crawling tail-end forward through paternal tentacle during spawning. In the diagram, a spermatozeugma is moving from left to right. Reverse waveforms are being generated from the tip to the base of axonemes. Reverse waveforms push against the inner walls of the tentacle (arrowheads) and move the spermatozeugma tail-end forward.

In *M. membranacea*, spermatozeugmata also generate reverse waveforms after contacting an ITO. The ITO is oriented with its distal pore directed away from the funnel of the lophophore. Consequently, spermatozeugmata within lophophores cannot be oriented to enter maternal individuals unless they develop a bend. A spermatozeugma undergoing a reverse-waveform event develops a strong conformational change that curves the head end of an aggregate relative to its long axis. This conformation change bends the spermatozeugma toward the distal pore of the ITO. Once the head end of a sperm aggregate is inside an ITO, the spermatozeugma likely changes its waveform dynamics to generate a waveform that pulls it forward.

The generation of reverse waveforms by a spermatozeugma of *M. membranacea* after contacting an ITO may be a response to a substance either on the distal surface or emanating from the pores of the ITO that acts as a sperm chemoattractant. To date, no sperm chemoattractants have been identified for gymnolaemate bryozoans (see Reed, 1991). Nevertheless, the change in waveform dynamics at the ITO may be evidence for a behavioral response of gymnolaemate bryozoan sperm to contacting maternal tissue of conspecifics. Although external fertilization has been reported among gymnolaemate bryozoans (*e.g.*, Silén, 1966), all studies that have actually confirmed the presence of a sperm or male pronucleus in oocytes indicate that sperm-egg fusion occurs before or during ovulation (see Ryland and Bishop, 1993; Temkin, 1994, 1996).

Internal fertilization in gymnolaemate bryozoans probably involves the entry of spawned sperm into the maternal coelom, and is not the result of eggs and sperm being produced in the same perivisceral coelom by hermaphro-

ditic zooids (see Ryland and Bishop, 1993; Temkin, 1994, 1996). Consequently, it is essential for spawned sperm to recognize the entryway into the maternal coelom, such as the ITO in *M. membranacea*. The presence of a sperm chemoattractant was also suggested by Silén (1966), based on his observations that sperm of *Electra posidoniae* attached to the abfrontal sides of tentacles began to produce "violent jerks" to move to the distal opening of the ITO at the time of egg spawning. ITOs have been reported to develop in species of seven gymnolaemate genera: *Membranipora, Electra, Alcyonidium, Conopeum, Farella, Victorella,* and *Bulbella*. Among these species, sperm entry through the ITO has been confirmed only for *M. membranacea* (Temkin, 1994). Silén (1966) reported that sperm entered the ITO of *Electra crustulena,* but he did not determine where sperm-egg fusion occurred. In species without ITOs, the supraneural pore, which represents the proximal pore of the ITO, has been hypothesized to serve as the entryway for sperm into the maternal coelom (see Silén, 1966). However, sperm entry through the supraneural pore has yet to be documented for any species.

Since spermatozeugmata are only known to occur in the gymnolaemate genera of *Membranipora* and *Electra,* the waveforms so far described for *M. membranacea* spermatozeugmata may not necessarily be produced by sperm of other gymnolaemate species. In fact, the waveforms produced by spermatozeugmata of *Electra* remain to be determined. However, some initial data on the waveforms generated by sperm of *Thalamoporella floridana* suggest that the waveforms observed for *M. membranacea* spermatozeugmata, including reverse waveforms, do occur in other gymnolaemate bryozoan sperm (Temkin, 2001). To understand the significance and evolution of spermatozeugmata and the waveform dynamics of gymnolaemate bryozoan sperm, further studies are required to determine how sperm locate, identify, and enter maternal individuals in gymnolaemate species that do not spawn spermatozeugmata or do not form ITOs.

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